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Research paper

Tolerogenic nanoparticles to induce immunologic tolerance: Prevention and reversal of FVIII inhibitor formation

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ABSTRACT

The immune response of hemophilia A patients to administered FVIII is a major complication that obviates this very therapy. We have recently described the use of synthetic, biodegradable nanoparticles carrying rapamycin and FVIII peptide antigens, to induce antigen-specific tolerance. Herein we test the tolerogenicity of nanoparticles that contains full length FVIII protein in hemophilia A mice, focusing on anti-FVIII humoral immune response. As expected, recipients of tolerogenic nanoparticles remained unresponsive to FVIII despite multiple challenges for up to 6 months. Furthermore, therapeutic treatments in FVIII-immunized mice with pre-existing anti-FVIII antibodies resulted in diminished antibody titers, albeit efficacy required longer therapy with the tolerogenic nanoparticles. Interestingly, durable FVIII-specific tolerance was also achieved in animals co-administered with FVIII admixed with nanoparticles encapsulating rapamycin alone. These results suggest that nanoparticles carrying rapamycin and FVIII can be employed to induce specific tolerance to prevent and even reverse inhibitor formation.

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1. Introduction

Replacement therapy for monogenic diseases, such as hemophilia A and B [1,2] and Pompe Disease [3], is complicated by the adverse immune response against the therapeutic protein [4]. For example, 25–30% of hemophilia A patients develop anti-FVIII inhibitory antibodies (inhibitors), which render the life-saving therapy itself ineffective [5]. The major approach to reverse inhibitor formation is “immune tolerance induction (ITI)” therapy, a protocol that requires regular high dose FVIII infusion for months to years. ITI is costly and fails in a significant number of patients, especially those with high titer inhibitors [6]. Hence, novel and durable antigen-specific tolerogenic therapy would be highly desirable from an efficacy and safety perspective.

Multiple techniques for antigen-specific immunotherapy for undesirable immune responses have been described, primarily in pre-clinical models, but translation to human clinical trials has been limited [7]. Strategies to induce tolerance include conjugating antigen to splenocytes [8], immature dendritic cells (DC) [9,10], or synthetic microparticles [11,12], oral tolerance [13], gene therapy

[14] or co-treatment with immunosuppressive drugs, such as methotrexate [15]. Recently, it has been reported that systemic co-administration of rapamycin, an immunosuppressant drug commonly used to prevent transplant rejection, prevented immune response and induced tolerance to FIX and FVIII in mouse models of hemophilia A and B, respectively [29,30].

Our goal was to develop a simple and effective protocol to induce tolerance to FVIII, both prophylactically and therapeutically. Based on the advantage of using a therapeutic nanoparticle delivery system [16–19], as well as the evidence that rapamycin treatment promotes tolerance induction both *in vitro* and *in vivo* [10,21,29,30], we developed tolerogenic synthetic vaccine particles (SVP) by incorporating rapamycin in self-assembling, biodegradable nanoparticles comprised of poly(lactic-co-glycolic acid) (PLGA), a polymer found in multiple medical products [20]. The use of these tolerogenic nanoparticles incorporating rapamycin could eliminate the need for chronic use of immunosuppressive systemic drugs.

Herein, we show that co-administration of SVP with FVIII led to specific unresponsiveness that was long-lasting and effective in FVIII knockout mice in a prophylactic protocol even with multiple challenges with immunogenic doses of FVIII. Moreover, an ongoing immune response in FVIII-primed mice could also be reversed by these SVPs, resulting in correction of bleeding phenotype following FVIII infusion.

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2. Materials and methods

2.1. Reagents

NPs were prepared as described and provided courtesy of Dr. Kei Kishimoto, Selecta Biosciences [20]. Briefly, for preparing SVP containing rapamycin and FVIII protein (Fig. 1A), PLGA, PLA-PEG, and rapamycin were dissolved in dichloromethane to form an oil phase. An aqueous solution of recombinant human FVIII (Advate, Baxalta) was then added to the oil phase and emulsified by sonication. Following emulsification of the antigen solution into the oil phase, a double emulsion was created by adding an aqueous solution of polyvinyl alcohol and sonicating a second time. The double emulsion was added to a beaker containing phosphate buffer solution and stirred at room temperature for 2 h to allow the dichloromethane to evaporate. When creating SVP contains rapamycin but no antigen (Fig. 3A), or NPs without any encapsulated agents, a similar oil-in-water single emulsion process was used. The resulting NPs were washed by centrifuging at 75,600g and 4 °C followed by re-suspension of the pellet in PBS. Tolerogenic NP injections typically consisted of 30–100 µg of rapamycin content.

Recombinant human FVIII (Baxalta) was used for immunization intravenously or intraperitoneally at 1 µg per injection; ϕ X174 was provided by Dr. Hans Ochs (Seattle Children's Hospital). Sheep red blood cells (SRBC) were purchased from Innovative Research, Inc., (Novi, MI), and 2,4,6-Trinitrophenyl conjugated SRBC was prepared as described [23].

2.2. Animals

FVIII^{-/-} mice (E16) on C57BL/6 background were originally obtained from Dr. Leon Hoyer at the American Red Cross [24]. All animals were housed and bred at the animal facilities operated by the Uniformed Services University of the Health Sciences (USUHS) Laboratory Animal Medicine facility, and animal protocols were approved by the Institutional Animal Care and Use Committee of USUHS.

2.3. Bleeding assay

Phenotypic correction of hemophilia A mice in response to therapeutic FVIII was assessed using a tail bleeding assay with modification [22]. Hemophilia A mice were injected with 1 µg FVIII in 100 µl PBS through the retro-orbital sinus. Five minutes after the injection, the tail tip was transected using a 1.6 mm diameter template without subsequent cauterization. Fifty microliter heparinized blood was collected through retro-orbital bleeding before the FVIII injection (0 h time point) and 6 h after the tail tip transection (6 h time point). Hemoglobin (Hb) levels in the blood were measured using Drabkin's reagent. Briefly, 4 µl of whole blood was mixed into 1 ml of Drabkin's solution at room temperature, and the absorbance at 540 nm was measured 15 min later. A standard curve was generated using bovine hemoglobin standards. The Hb levels at 6 h time point were normalized to the value at 0 h, and the result was reported as % normalized hemoglobin. The same protocol was also performed on a group of wild type C57BL/6 mice (WT), except without FVIII injection.

2.4. Immunologic assays

FVIII activity was assayed using the chromogenic functional assay (Coatest SP4 FVIII, DiaPharma). ELISA and Bethesda assays for measuring anti-FVIII IgG titer and for the FVIII inhibitor titer, respectively, were performed as previously described [14,25]. Anti-trinitrophenyl and anti- ϕ X174 titers were performed by

ELISA on haptenated-BSA and ϕ X174-coated plates, respectively. Anti-SRBC titer was determined by hemagglutination (HA) assay as described [26].

2.5. Statistics

Normal distribution data were compared using a 2-tailed unpaired Student's *t* test. If the data did not meet the Shapiro–Wilk test for normality, the Mann–Whitney *U* test was performed. Data are presented as mean \pm SEM, or otherwise as indicated. A *p* value of less than 0.05 was considered significant.

3. Results

3.1. Prophylactic treatment in hemophilia A mice

To test the efficacy of SVP containing FVIII protein and rapamycin, we first treated naïve E16 mice. These mice have a targeted disruption of FVIII exon 16 which results in an undetectable level of FVIII activity; such E16 mice have been used as a small animal model for severe hemophilia A [27]. E16 mice (*n* = 8–10 animals/group) were treated weekly for 5 weeks with test SVP or control empty NP through *i.v.* route (Fig. 1B). For the last three doses, the mice were co-administered with an immunogenic dose of FVIII. As expected, mice treated with empty nanoparticle (empty NP) control showed a robust anti-FVIII antibody response with a mean level of 18.4 µg/ml (4.2–45.8 µg/ml), one week after the 3rd FVIII exposure. In contrast, the anti-FVIII titer was undetectable in mice treated with 5 doses of SVP. To confirm the unresponsiveness to FVIII, the mice were challenged again with FVIII on day 21. Three weeks later at day 42, 7 out of 8 mice in the SVP group remained undetectable for anti-FVIII titer, while the anti-FVIII level in the control group continued increase to a mean level of 34.6 µg/ml (Fig. 1C).

3.2. Tolerance to FVIII induced was long-lasting

The mice were rested for additional 3 weeks and then challenged with 3 more weekly injections of immunogenic doses of FVIII. As shown in Fig. 1C, the control group mice (black dots) further responded with peak mean levels of anti-FVIII at 49.1 µg/ml. Following the same treatments, the peak mean anti-FVIII levels were 14.8 µg/ml in SVP group. Removal of one outlier resulted in a peak mean anti-FVIII levels of only 1.9 \pm 0.6 µg/ml (0–3.2 µg/ml), which was 25-fold less than that of control group. The anti-FVIII inhibitor titer showed a similar trend (Fig. 1D). On day 65, a bleeding assay was performed. A group of wild type (WT) C57BL/6 mice were included in order to establish base line. As shown in Fig. 1G, the blood loss in WT mice resulted in normalized Hemoglobin (Hb) of 78.1 \pm 5.3%. FVIII injection in the empty NP group mice failed to rescue the hemophilia phenotype of E16 mice, due to the presence of high titer anti-FVIII inhibitors. The normalized Hb levels were 63.3 \pm 2.1% in the empty NP group, significantly lower than that of WT group. In the contrast, administration of FVIII in SVP-treated animals 5 min before the bleeding assay procedure completely rescued the hemophilia phenotype, resulting in 85.9 \pm 5.9% normalized Hb levels.

3.3. Normal antibody response to TNP antigen

Tolerance to FVIII developed in the SVP group was antigen-specific, as these mice responded normally to immunization with a non-related antigen, TNP (Fig. 1F). The mice received three weekly *i.v.* injections of TNP-SRBC between day 42 and 58. As early as one week after the 1st TNP-SRBC dose, the majority of the mice in both groups responded with anti-TNP antibodies, which further

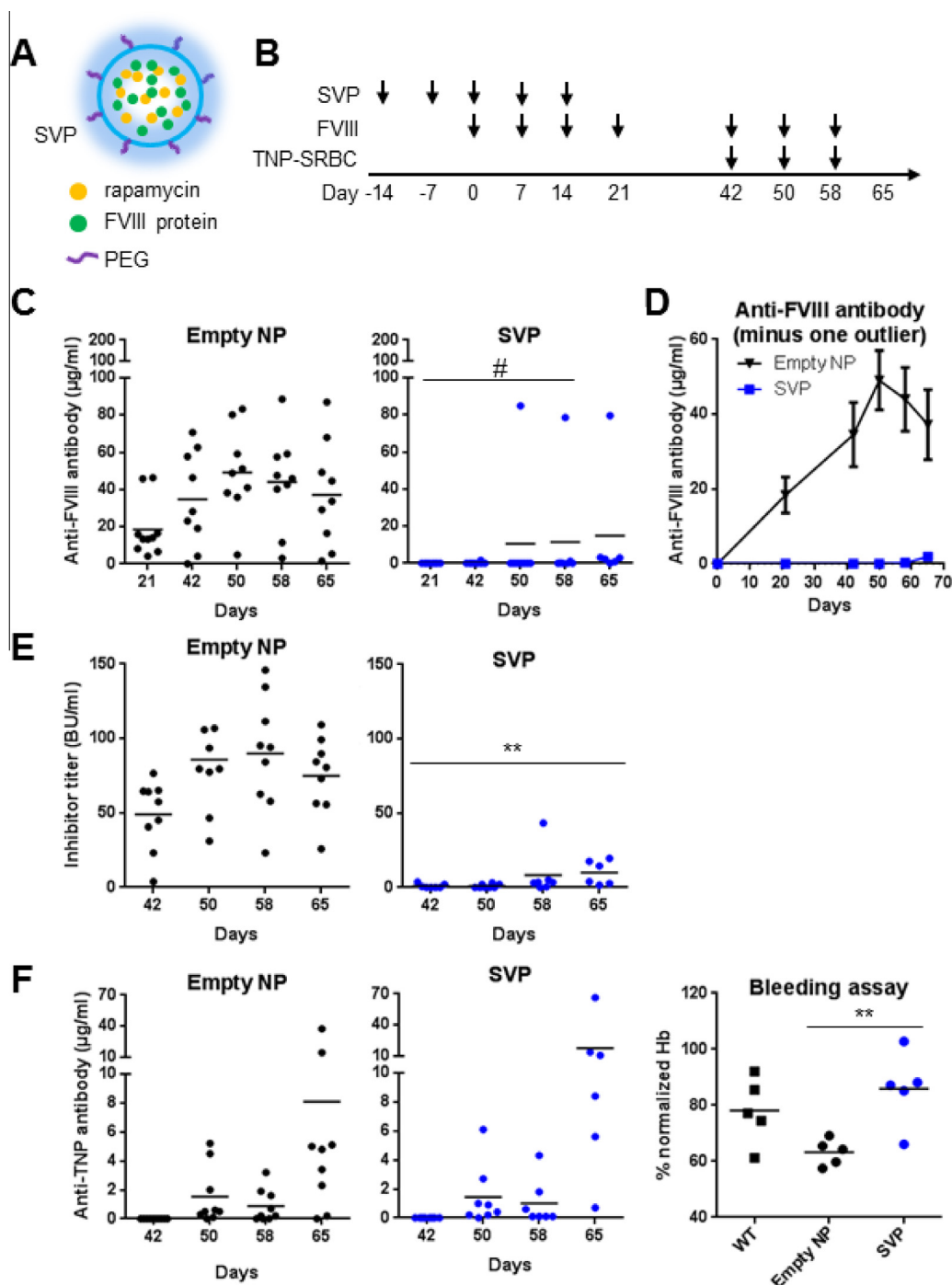


Fig. 1. Prophylactic efficacy of SVP in E16 mice. (A) Illustration of SVP encapsulating with both rapamycin and full length FVIII protein. (B) Dose regimen. E16 mice were treated with either SVP or control empty NP through i.v. on days -14, -7 and 0. Both groups received recombinant human FVIII (1 µg) on days 0, 7, 14, 21, 42, 50, and 58 and were also immunized with trinitrophenol-conjugated sheep red blood cells (TNP-SRBC) on days 42, 50 and 58. (C) Anti-FVIII antibodies were determined by ELISA. (D) Anti-FVIII antibodies in animals treated with 5 doses of empty NP (black) vs SVP (blue) after removal of a single outlier from the SVP group. (E) FVIII inhibitor titers were determined by measuring Bethesda Units. (F) Anti-TNP-SRBC antibodies were determined by ELISA. (G) The correction of bleeding phenotype in the E16 mice as revealed by % normalized hemoglobin (Hb) in a bleeding assay. # $p < 0.05$ compared to empty NP group by Mann Whitney U test with two tailed distribution; * $p < 0.05$ and ** $p < 0.01$ compared to empty NP group by Student t test with two tailed distribution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

increased with additional injections. There was no significant difference between the two groups at any time point.

3.4. Therapeutic treatment of an emerging anti-FVIII immune response

We next assessed the ability of SVP to therapeutically inhibit an emerging immune response in E16 mice primed with FVIII

(Fig. 2A). Mice received 4 injections of low dose FVIII (0.1 µg) on days 0, 7, 14, and 25 followed by two doses of high dose FVIII (1 µg) on days 34 and 42. FVIII inhibitor titers were assessed on days 25, 34, 42, and 51. Animals that had low but detectable levels of inhibitors on Day 51 were randomized to either empty NP or SVP treatment (Fig. 2B). Animals received two courses of 5 weekly injections of nanoparticles concurrently with FVIII (1 µg) starting

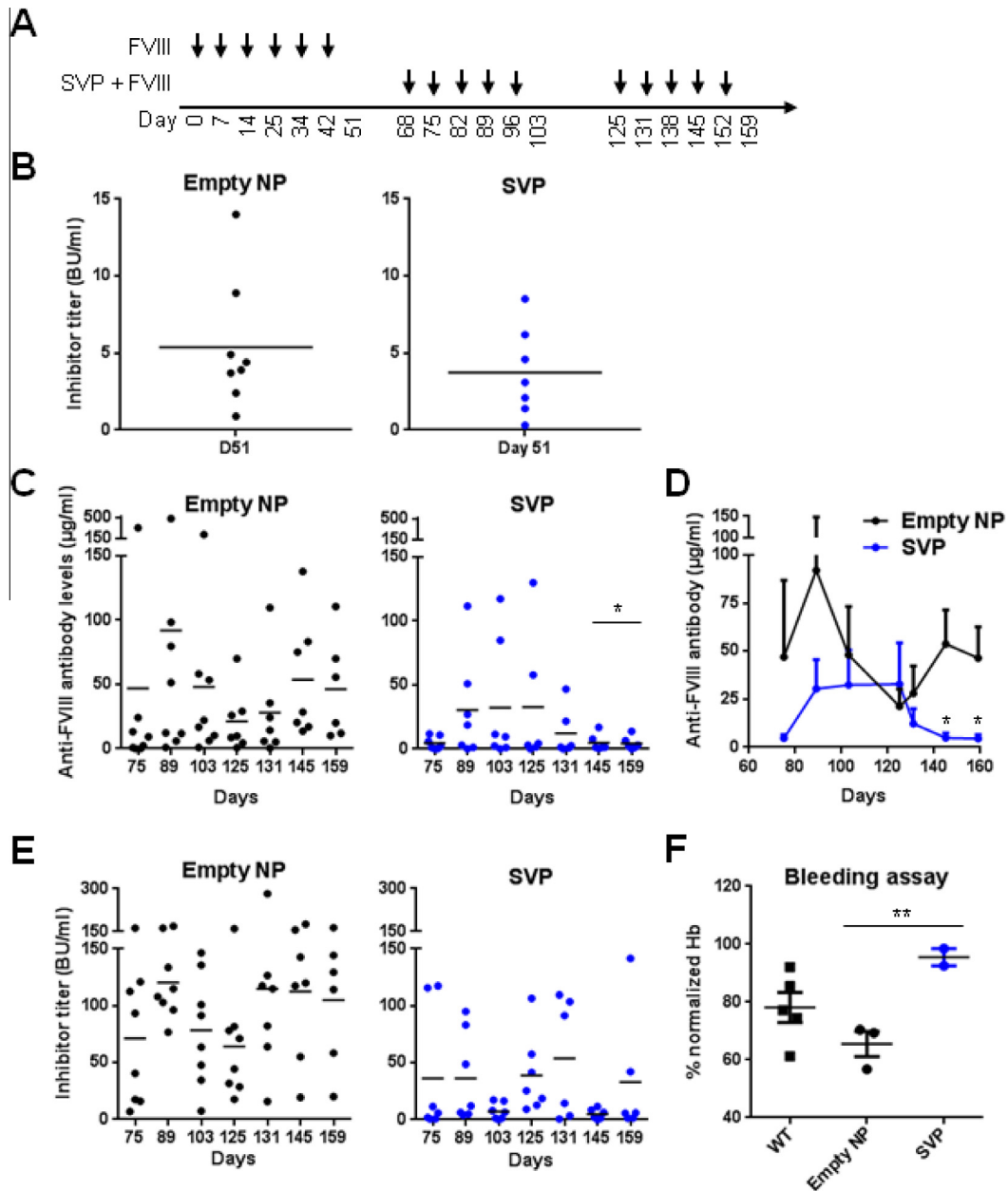


Fig. 2. Therapeutic efficacy of SVP in FVIII primed mice. (A) Dose regimen. E16 mice were received 4 priming doses of 0.1 µg FVIII on days 0, 7, 14, and two doses of 1 µg FVIII on days 34 and 42. Animals were administered two courses of 5 weekly treatments with either control empty NP or SVP concurrently with FVIII. (B) Day 51 pretreatment FVIII inhibitor titers were determined by measuring Bethesda Units. (C) Anti-FVIII antibodies were determined by ELISA. (D) Anti-FVIII antibodies in animals treated with empty NP (black) vs SVP (blue). (E) Day 75–159 FVIII inhibitor titers were determined by measuring Bethesda Units. (F) The correction of bleeding phenotype in the E16 mice as revealed by % normalized hemoglobin (Hb) in a bleeding assay. * $p < 0.05$ and ** $p < 0.01$ compared to empty NP group by Student t test with two tailed distribution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

on days 68 and 125 (Fig. 2A). Animals treated with empty nanoparticle control showed a variable but significant anti-FVIII antibody response (Fig. 2C and D, black symbols). Mice treated with SVP initially showed a variable response to FVIII, with some animals exhibiting high levels of anti-FVIII antibodies while other animals remained low (Fig. 2C, blue symbols). Importantly, after the start of the second course of treatment on Day 125, animals treated with SVP showed a substantial reduction in antibody levels (Fig. 2C and D, blue symbols). Analysis of FVIII inhibitors showed a similar trend (Fig. 2E), although there was more variability in the levels of inhibitors between time points in the SVP group. A bleeding assay was performed in some of the mice on day 216, about 2 months after the final concurrent dose of nanoparticles and FVIII. Compared to the WT group ($78.1 \pm 5.3\%$, see Fig. 1G), the

normalized Hb levels in empty NP and SVP groups were $65.5 \pm 4.4\%$ and $95.6 \pm 3.0\%$, respectively (Fig. 2F).

3.5. Tolerance induction using SVP that contains rapamycin only

Experiments were performed in E16 mice to determine the efficacy of SVP that contains rapamycin only (Fig. 3A), since we observed that FVIII protein was able to physically bind to the SVP after mixing together (Fig. 3B). To ensure NP mediated co-delivery of FVIII antigen and rapamycin to the same antigen-presenting cells, FVIII was diluted in PBS and then mixed with an equal volume of NPs before the treatment was administered to the animals in these experiments.

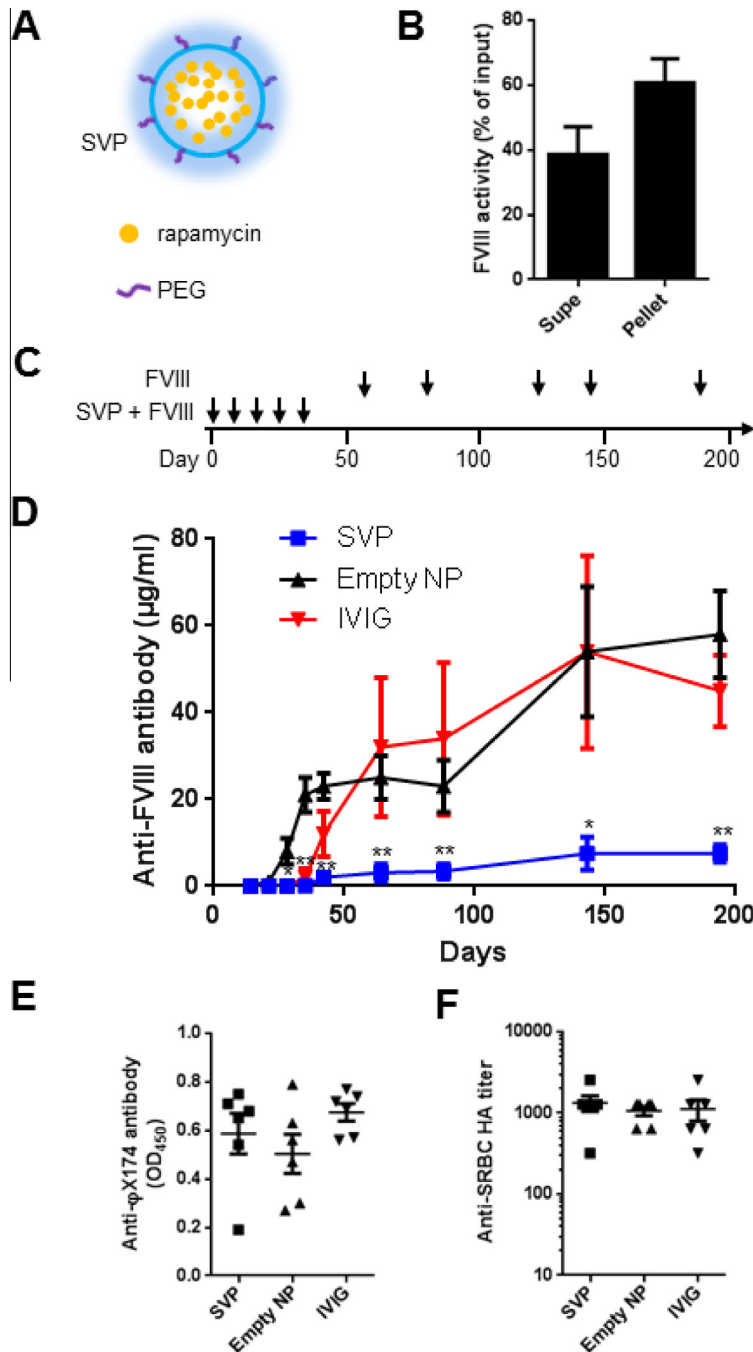


Fig. 3. Prophylactic treatment for tolerance induction using SVP that contains rapamycin only. (A) Schematic illustration of SVPs encapsulating rapamycin alone. (B) FVIII binds to SVP *in vitro*. Recombinant human FVIII (1 μg, diluted in 100 μl PBS) was added to 100 μl of F2 SVP and mixed by brief vortex. After incubation at room temperature for 10 min, the mixture was separated by centrifugation at 16,000g for 10 min at 4 °C. The pellet was reconstituted in 200 μl PBS. The FVIII activity of the supernatant (supe) and of the reconstituted pellet (pellet) was immediately measured by chromogenic method. About 99% of the input FVIII activity was recovered. The result was expressed as percentage of the input FVIII activity (mean ± SEM). (C) Dose regimen. Naïve E16 mice ($n = 8$) received weekly *i.v.* injection for 5 weeks with the mixture containing 100 μl of FVIII (1 μg) in PBS plus 100 μl of either SVP, empty NP or IVIG (1 mg). After the final SVP treatment, the mice received multiple FVIII only challenge (1 μg) via *i.v.* or *i.p.* at day 57, 81, 125, 143, and 187, respectively. Blood samples were obtained one week after each FVIII exposure, and anti-FVIII antibody levels in the sera were determined by ELISA. (D) Anti-FVIII antibodies in animals treated with SVP (blue), empty NP (black) or IVIG (red). (E) and (F) Antigen specificity of the tolerance effect. The mice described were immunized through *i.v.* with 2.5×10^8 pfu of φX174, a non-relevant antigen, at day 81, 95, and 143, respectively. At day 158, fifteen days after the last phage immunization, blood samples were obtained, and the anti-φX174 antibody levels were determined by ELISA. In addition, the mice were also *i.v.* challenged with another non-relevant antigen, sheep red blood cells (SRBC), at day 115 and 170, respectively. The anti-SRBC antibody titer in the serum one week after the second SRBC immunization was determined by hemagglutination assay (HA). (E) Anti-PhiX174 antibody levels measured by ELISA. (F) The anti-SRBC HA titer. * $p < 0.05$ and ** $p < 0.01$ compared to empty NP group by Student *t* test with two tailed distribution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

To test the ability of SVP containing only rapamycin to induce tolerance prophylactically, groups of naïve E16 mice were injected with immunogenic doses of FVIII admixed with either SVP or empty NP, while a third group received FVIII plus a high dose of

intravenous immunoglobulin (IVIG), which is known to be immunosuppressive (Fig. 3C).

After four weekly injections, all the mice in the control group receiving empty NP + FVIII developed high titer anti-FVIII

antibodies. The SVP treatments effectively prevented anti-FVIII antibody development throughout the course of the 5-week treatment. The majority of anti-FVIII antibodies developed in mice receiving empty NP + FVIII treatments was of IgG1 subtype. Inhibitor titers were also determined which followed the same trend as the antibody titers (Table 1). The group receiving IVIG plus FVIII similarly developed low anti-FVIII antibody titers during the treatment phase. However, upon cessation of treatment and further challenge with FVIII, the IVIG-treated group developed titers similar to those animals that received empty NP plus FVIII (Fig. 3D).

In contrast, those mice that received SVP remained hyporesponsive to multiple challenges with FVIII. Remarkably, the FVIII tolerance state in the mice that received SVP plus FVIII treatments was sustained for over 6 months despite multiple injections with immunogenic doses of FVIII (Fig. 1D). These results indicate that the IVIG induced only transient immunosuppression during the period of treatment, while the SVPs induced durable immune tolerance.

To rule out that SVP are inducing a chronic state of immunosuppression, we immunized the mice with two additional unrelated antigens, bacteriophage ϕ X174 and SRBC, both of which are immunogenic without additional adjuvants, like FVIII. The two groups produced similar levels of antibody responses to these non-relevant antigens, indicating the efficacy of SVP + FVIII prophylactic therapy was antigen-specific (Fig. 3E).

Although prevention of an immune response to FVIII was achieved, reversal of antibody formation is more challenging and relevant to FVIII inhibitor management. To test SVP in a therapeutic protocol, E16 mice were injected weekly with recombinant human FVIII to induce anti-FVIII antibody responses. The animals were then distributed in a blinded manner so that the treatment groups contained equal numbers of animals with similar titers. Animals then received two courses of 5 weekly injections of SVP containing rapamycin admixed with FVIII or empty NP plus FVIII (Fig. 4A). During the first course of treatment, animals that received SVP showed only a modest reduction in titers compared to the control. However starting with the second course of treatment, on Day 85, the SVP-treated mice showed a marked reduction in anti-FVIII titers while the control animals showed a substantial boost in titers. By day 113, mice treated with SVP plus FVIII were significantly suppressed for anti-FVIII antibody production (Fig. 4C). This effect also was specific as both groups responded to TNP-SRBC (data not shown).

4. Discussion

4.1. SVP approach for FVIII immune tolerance

The immune response to therapeutic FVIII in hemophilia A is the major factor rendering this therapy ineffective and potentially life-threatening. Despite the wide use of ITI in treating inhibitor formation, this approach is very expensive, involves long-term treatment and is not always effective. To approach tolerance induction to FVIII, we employed nanoparticles that have been shown in other models to induce antigen-specific immune tolerance [20]. The tolerogenic nanoparticle (SVP) are made with a polymer, PLGA, that is biocompatible, biodegradable and used in multiple products licensed for clinical use by regulatory agencies [28]. We have combined it with an immunomodulatory agent, rapamycin, which has been validated in humans and is capable of facilitating tolerance [10,20,21,29,30]. The delivery of rapamycin by nanoparticles allows for efficient transport to lymphoid organs and capture by phagocytic antigen presenting cells (APCs) [20], and thus reduce non-specific toxicity through chronic systemic administration. It is of note that the weekly dose of rapamycin used in the SVP

Table 1

The effect of prophylactic treatment of SVP on antibody response to co-delivered FVIII: two weeks after the final SVP treatment and before challenge.

	Inhibitor titer (BU/ml)	Anti-FVIII antibodies (μ g/ml)		
		Total Abs	IgG1	IgG2a
Empty NP	92.9 \pm 2.5	23.34 \pm 2.99	13.04 \pm 2.87	1.78 \pm 0.40
SVP	14.3 \pm 7.2**	2.01 \pm 1.11**	1.34 \pm 0.80**	0.34 \pm 0.05**
IVIG	30.3 \pm 5.6**	12.07 \pm 5.22	1.33 \pm 0.53*	1.84 \pm 1.01

* $p < 0.05$ and ** $p < 0.01$; compared to empty NP group using Student-*t* test with two tailed distribution.

approach was more than 6-fold less than in systemic delivery reported by others [29,30]. For maximum efficacy, the dose and frequency of SVP treatment will need to be further optimized in future studies.

4.2. Different formulations of SVP

We previously reported that SVP encapsulating rapamycin as well as a mixture of three MHC class II-binding FVIII peptides induced specific tolerance to co-administered FVIII [20]. In this study, we found that co-delivery of FVIII plus SVP containing rapamycin only was equally effective, if not better, than SVP containing both rapamycin and FVIII protein. This was attributed to the fact that FVIII was able to bind to SVP when mixed together. Since the SVP encapsulating FVIII peptides in previous report was also administered together with FVIII, it could not be rule out that part of the efficacy might be due to FVIII binding onto the SVP. However, other protein antigens, e.g. OVA, did not bind to SVP in significant amount (data not shown). Moreover, SVP containing rapamycin could induce tolerance to other antigens, even when the nanoparticles and the antigen were administered in separate injections (RAM and TKK, unpublished data). The advantage of using the SVP containing rapamycin alone is that it can be simply added on to normal FVIII therapy in the clinic. However a potential advantage of using the SVP that contains both FVIII and rapamycin is that it could be used as a prophylactic vaccine to render patients tolerant to FVIII prior to their first treatment. In the United States, it is common for newly diagnosed hemophilia A patients to not receive FVIII replacement therapy until there is a bleeding episode that requires its use. Thus there is a window in which a newly diagnosed infant could be tolerized to FVIII with SVP prior to the first therapeutic use FVIII therapy. The advantage of encapsulating the entire protein, rather than selected MHC class II-binding peptides, is the dominant MHC class II peptides will vary from individual to individual because of MHC class II heterogeneity, whereas the FVIII protein contains all possible MHC class II-binding epitopes.

4.3. Outlier response

The outcome of successful tolerance induction may depend on the sum of many factors in each individual, including the ongoing inflammation and/or infection. While the general trend of a potential tolerogenic efficacy is clear in this study, we did see several outlier responses, e.g. in Fig. 1C and D. It is less likely that tolerogenic SVP was directly responsible for the outlier response, considering the fact that SVP was not immunogenic, and up to four weeks after the final prophylactic exposure to SVP there was still no measurable anti-FVIII antibody response developed. While determining the exact cause of lack of tolerance in individual animal is important, it was out of the scope of our current study, and it should not affect the main conclusion drawn.

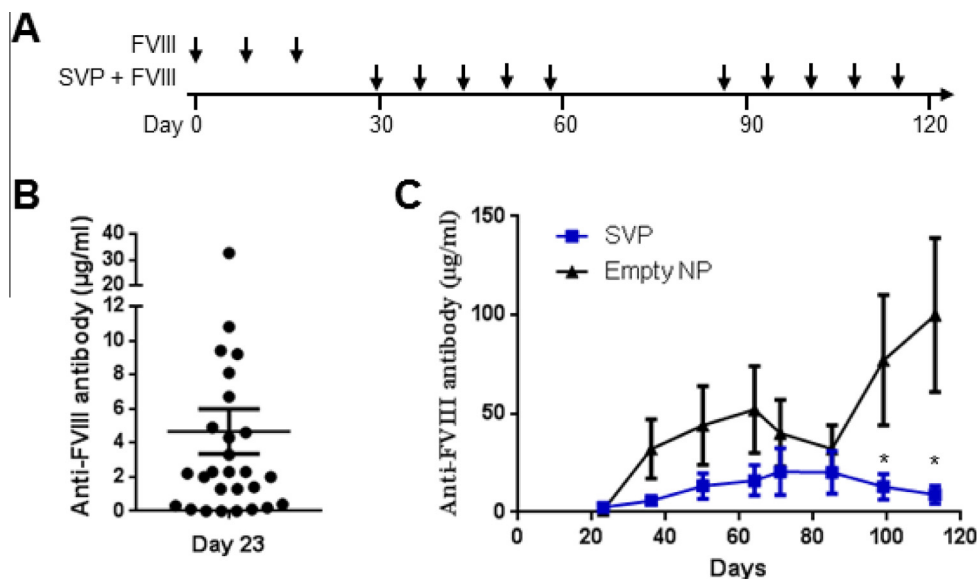


Fig. 4. The therapeutic treatment for tolerance induction using SVP that contains rapamycin only. (A) Dose regimen. A group of E16 mice ($n = 27$) were primed with three times weekly i.v. injection of $1 \mu\text{g}$ rFVIII in $100 \mu\text{l}$ PBS. On day 23, one week after the 3rd injection, anti-FVIII antibody levels were determined by ELISA (B). The primed E16 mice were then assigned into three treatment groups ($n = 8$ or 7 at day 23). Starting on day 29, each group of mice received two rounds of five weekly concomitant NP plus FVIII treatments. (B) The anti-FVIII antibody levels after three priming doses. The majority of the E16 mice developed detectable anti-FVIII antibodies, at level of $4.7 \pm 1.3 \mu\text{g/ml}$. (C) Anti-FVIII antibody levels in mice treated with empty NP (black) or SVP (blue). $p < 0.05$; compared to empty NP group by Student t test with two tailed distribution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.4. Mechanism of action

The precise mechanism of action by SVP remains to be determined. We know that antigen-presenting cells (APC) are attractive targets for immunotherapies due to their central role in antigen presentation to T cells and their ability for inducing tolerance. Nanoparticles are ideal vehicles to deliver antigen and drugs to APCs, as these cells are efficient in capturing particulate antigen, such as viruses. Nanoparticles injected are rapidly taken up by antigen-presenting cells, such as macrophages and dendritic cells [17–20]. The nanoparticle delivery of rapamycin and antigen to the same APC may facilitate tolerogenic antigen presentation. Indeed, SVP treatment inhibited expansion of antigen-specific T cells and induced antigen-specific regulatory T cells (Treg) [20]. The total number of Tregs may not necessarily be increased. We have looked at the frequency of total splenic Foxp3 + Tregs among the CD4+ T cell populations by flow at the end of the experiment described in Fig. 1 and did not find significant difference between the two groups (data not shown). In future studies, among others, functional activity of the induced specific Tregs by SVP treatment will need to be assessed by adoptive transfer experiments.

4.5. Conclusion

We report in this study a new and effective method for immune tolerance induction to therapeutic FVIII by utilizing PLGA nanoparticles carrying immune modulating agent rapamycin as well as the target antigen, FVIII protein. The addition of tolerogenic nanoparticle strategy to FVIII replacement therapy has the potential to improve the efficacy and safety profile of FVIII in the treatment of hemophilia patients, either for prophylaxis or for purpose of managing bleeding.

Addendum

A.H.Z., J.Y., and D.W.S. designed the experiments. A.H.Z., R.J.R., J.Y., and H.W. performed the experiments. AHZ and DWS wrote the paper.

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